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Quantification of methane oxidation in the rice rhizosphere using ^{13}C-labelled methane

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Abstract. In this paper isotope ratio mass spectrometry is used to determine the methane (CH_4) oxidation fraction in the rhizosphere of intact rice plant-soil systems. Earlier studies on quantification of the methane oxidation were based on inhibition or incubation procedures which strongly interfered with the plant-soil system and resulted in a large variability of the reported fractions, while other studies considered stable isotopes at natural abundance levels to investigate methanotrophy in the rhizosphere of rice. The current work is the first that used ^{13}C-labelled CH_4 as additive and calculated the oxidation fraction from the ratio between the added ^{13}C-labelled CH_4 and its oxidation product $^{13}CO_2$. Both labelled gases could be distinguished from the natural abundance percentages. The oxidation fraction for methane was found to be smaller than 7%, suggesting that former approaches overestimate the methane oxidation fraction.

Introduction

Methane is the third most important greenhouse gas, after CO_2 and H_2O. Moreover, it takes the second place in the group of greenhouse gases which are seriously influenced by the anthropogenic activities. Its concentration has risen from 0.7 ppm in the pre-industrial period to 1.7 ppm in 1997 (Steele et al. 1992) and at present it is responsible for 20% of the radiative forcing (Ramaswamy 2001). The contribution of rice paddies to the total emission of methane (530 ± 20 Tg per year) is considerable but not known precisely (Prather et al. 1995). This uncertainty is partially caused by the large variations in local rice growth conditions and by the complicated dynamics between the methane production and methane oxidation in the rice paddy soil (van Bodegom et al. 2001). Therefore, a better understanding of methane oxidation in the rice rhizosphere is necessary. Under anaerobic conditions, methanogenic bacteria produce methane in the paddy soil. Their production de-

pends highly upon the availability of degradable organic matter. Methanogens use acetate (CH_3COOH) and H_2/CO_2 derived from organic material as substrates:

$$CH_3COOH \rightarrow CH_4 + CO_2$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

The first reaction path accounts for 70–90% of the methane production (Achtnich et al. 1995; Conrad 1993; Minoda and Kimura 1994). Methane production is fuelled by exudates of the roots (Chanton et al. 1997) and is highest at the end of the growing season when the roots are completely developed (Bilek et al. 1999; Minoda and Kimura 1994; Denier van der Gon and Neue 1996). The start of methane production in wetland rice varies from a few days to several weeks after flooding the field, depending on the chemical and microbial conditions of the soil (Conrad 1993). Before flooding, wetland rice fields contain similarly high numbers of viable methanogenic bacteria as under anaerobic conditions (Conrad 1993). Thus, the onset of CH_4 production apparently does not depend on the growth in number of methanogenic bacteria.

Methane can escape from the rice paddy soil in various ways: via aerenchyma in the plant (90%), via ebullition (10%), or via diffusion through the soil and water layer (1%) (Conrad 1993; Denier van der Gon and Neue 1996). Ebullition dominates in unvegetated plots (Chanton and Dacey 1991). Methane transport via the plant starts in the roots; methane enters by diffusion through the epidermis and during the water uptake. It is likely that dissolved CH_4 is directly gasified in the root cortex (Nouchi 1994) and further diffuses upwards to the root-shoot transition zone via intercellular spaces and aerenchyma. After crossing the high diffusion barrier formed by the root-shoot transition zone (Butterbach-Bahl et al. 1997), methane is transported into the open atmosphere, mainly via the shoot aerenchyma and the micro pores of the leaf sheath and to a lesser extent via stomata (Nouchi 1994). The aerenchyma system is developed by the plant to transport the oxygen necessary for respiration towards the roots. Just like methane diffuses from the soil into the root system, oxygen diffuses from the root into the soil, creating a relative oxygen rich zone in the rhizosphere. Methane produced by methanogenic bacteria in the soil is partly oxidized in the rhizosphere to CO_2 by methanotrophic bacteria (Conrad 1993; Frenzel et al. 1992; Chanton et al. 1997; Bilek et al. 1999; Butterbach-Bahl et al. 1997). Methanogenesis in the rhizosphere itself is suppressed by oxygen (Fetzer and Conrad 1993).

To our knowledge, four approaches have been used to quantify methane oxidation in the rhizosphere, (1) soil core incubations, (2) the use of specific inhibitors to block methanotrophy, (3) inhibition of methanotrophy by using a N_2 atmosphere and (4) isotope ratio studies. In Figure 1 and Table 1 an overview is given of the reported results using different methods. As can be seen, the reported methane oxidation fractions show large variations.

Incubation studies compare the methane production from incubated soil samples with the methane emitted under field conditions. The difference between the two

Table 1. Methane oxidation fractions for rice plants measured by different techniques and reported by various authors. For all methods the days after flooding are given (see also Figure 1).

Inhibition			N_2 atmosphere		
Days after flooding	Oxidation percentage	Reference	Days after flooding	Oxidation percentage	Reference
50	31.4	(Banker et al. 1995)	49	3–30	(Bilek et al. 1999)
90	11	(Banker et al. 1995)	65	31–55	(Bilek et al. 1999)
All season	0–28	(Dan et al. 2001)	65	0–33	(Denier van der Gon and Neue 1996)
All season	0–33	(Dan et al. 2001)	65	44–48	(Denier van der Gon and Neue 1996)
37	22–34	(Denier van der Gon and Neue 1996)	All season	31–37	(Gilbert and Frenzel 1995)
65	0–27	(Denier van der Gon and Neue 1996)	All season	14–24	(Gilbert and Frenzel 1998)
65	0–6	(Denier van der Gon and Neue 1996)	??	42	(Holzapfel-Pschorn et al. 1985)
100	0	(Denier van der Gon and Neue 1996)	80	71	(Holzapfel-Pschorn et al. 1986)
90	7–52	(Epp and Chanton 1993)	44	3–31	(Sigren et al. 1997)
80	26	(Holzapfel-Pschorn et al. 1986)	57	31–55	(Sigren et al. 1997)
35	40	(Krüger et al. 2001)	28	38–68	(Wang et al. 1997)
65	15	(Krüger et al. 2001)	56	22–65	(Wang et al. 1997)
105	0	(Krüger et al. 2001)	84	14–68	(Wang et al. 1997)
Soil-core incubation studies			Isotope studies		
Days after flooding	Oxidation percentage	Reference	Days after flooding	Oxidation percentage	Reference
49	4–36	(Bilek et al. 1999)	36	12	(Bilek et al. 1999)
65	38–70	(Bilek et al. 1999)	49	13–33	(Bilek et al. 1999)
17	29–44	(Bosse and Frenzel 1998)	65	29–81	(Bilek et al. 1999)
115	17–38	(Bosse and Frenzel 1998)	90	0–94	(Chanton et al. 1997)
74	0–64	(Holzapfel-Pschorn et al. 1985)	38	12–48	(Tyler and Bilek 1997)
105	22–45	(Holzapfel-Pschorn et al. 1985)	56	7–58	(Tyler and Bilek 1997)
??	76–88	(Holzapfel-Pschorn et al. 1985)	77	25–59	(Tyler and Bilek 1997)
80	71–81	(Holzapfel-Pschorn et al. 1986)	86	41–67	(Tyler and Bilek 1997)
36	45	(Schütz et al. 1989)			
41	56–60	(Schütz et al. 1989)			
63	43–86	(Schütz et al. 1989)			
69	64	(Schütz et al. 1989)			

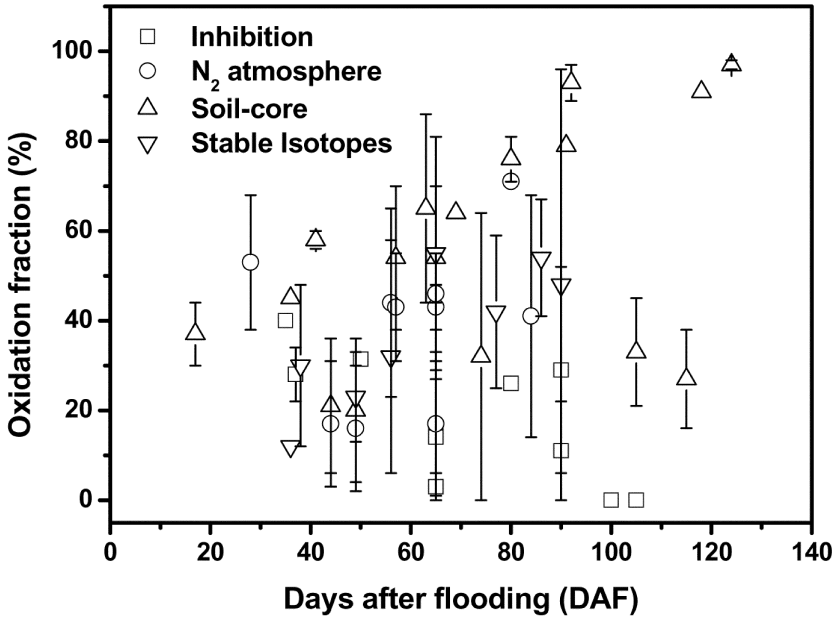

Figure 1. Methane oxidation fractions for rice plants as function of days after flooding, measured by different techniques (see also Table 1).

productions is taken as a measure for the amount of methane oxidised under field conditions. However, methane production rates may differ between field conditions and incubation studies. Conditions for methane production are optimal in the incubated soil, while methane oxidation does not occur. Furthermore, inhibition of methane production by oxygen does not occur. In addition, temperatures during incubation are usually higher than in the field, which would tend to increase methane production (Conrad 1993; Denier van der Gon and Neue 1996). On the other hand, carbon substrates from the rice plants are excluded from the incubated soil core, which might lead to an underestimation of total methane production. Most of the values reported for the fraction of methane that is oxidized by this method (Figure 1, Table 1) are above 50%, and they are higher than values obtained by the other methods. This suggests that incubation studies overestimate methane oxidation via an overestimation of methane production.

In the inhibition studies, inhibiting methane oxidation substances such as fluoromethane (CH_3F), difluoromethane (C_2H_2) or acetylene (CH_2F_2) are applied to the headspace around an enclosed plant. CH_3F completely inhibits methane oxidation and, depending on its concentration it also inhibits methanogenesis (Frenzel and Bosse 1996; Oremland and Culbertson 1992; King 1996). However, the effects of CH_3F on methane production from rice rhizosphere seem small during short-term incubations (Gilbert and Frenzel 1995). Acetylene is also a competitive inhibitor of methane oxidation; application of 0.01% of C_2H_2 reduced the methane oxidation by 90%, 0.1% by 96% and 1% by 98%, respectively (King 1996). Orem-

land and Culbertson (1992) found that at concentrations below 0.01%, acetylene reduced methane production by up to 60%. Watanabe et al. (1997) reported an inhibitory effect on methane production at this concentration only after long period of incubation, due to inhibition of the methanogens, while others found that 0.01% of C_2H_2 did not block pure cultures of methanogens (Oremland and Taylor 1975; Sprott et al. 1982). Inhibition of both methanogens and methanotrophs question estimates of methane oxidation by this method, but recently, methanotrophy was reported to be blocked specifically for a certain range of CH_2F_2 concentrations (Krüger et al. 2001).

Surrounding an enclosed plant completely with pure N_2 stimulated methanogenesis by 35% compared to plants surrounded by air (Denier van der Gon and Neue 1996) confirming the findings of Holzapfel-Pschorn et al. (1985). In consequence, the inhibition method leads to an overestimation of methane oxidation.

Finally, the methane oxidation percentage can be determined using the isotope ratio method. Coleman et al. (1981) measured the fractionation for methane oxidation by bacteria using $^{12}C/^{13}C$ ratios. Tyler and Bilek (1997) and Chanton et al. (1997), Bilek et al. (1999) calculated the methane oxidation fraction by comparing the methane isotope ratio from the plant with that in the soil water around the plant roots. Gas samples extracted from the lower part of the plant were assumed to have the same methane isotope ratio as in the epidermis of roots. Since gas in the lower part of the plant has crossed the root-shoot transition zone, with its high resistance for gas diffusion (Butterbach-Bahl et al. 1997) its has also undergone extra fractionation during diffusion. This can result in an overestimation of the oxidized methane percentage. Other sources which may provide inaccurate results are the uncertainties in isotope fractionation factors (Bergamaschi 1997) and in the background of non-oxidized $\delta^{13}C$-values. Depending on the assumption for these background values, Chanton et al. (1997) calculated methane oxidation in the range of 0 to 94%.

Clearly, all methods reported here have various drawbacks, most of which tend to lead to an overestimation of methane oxidation. Our approach was to add a small amount of ^{13}C-labelled methane, small compared to the amount of methane in the soil, to the root zone of a rice plant, and to trace the ^{13}C label after diffusing through soil, rhizosphere and aerenchyma system into the headspace around the plant. To determine the methane oxidation fraction, we measured both the release of $^{13}CO_2$ and $^{13}CH_4$, and corrected for the natural isotope ratio using reference plants. With this set-up several problems connected to the previous approaches are avoided.

Material and methods

Plant material

Rice plants of the high yielding cultivar IR72 were grown in a soil containing equal amounts of rice paddy field soil (Philippines) and river clay (the Netherlands). The

pH of the soil was 7.13. One-week-old seedlings were transferred to large plastic breeding trays containing this soil (six plants per tray, 23 cm between the plants). The plants were grown in the greenhouse under natural daylight conditions for 3 to 4 months (15 $MJm^{-2}d^{-1}$ for 1998) at 19 ± 1.5 °C. A water layer of 3 cm deep was kept on top of the soil during the cultivation. To stimulate the methane production, 10 g of acetate per plant was added to the water two to four weeks before measurements. The plants were transferred to glass jars 5, 17 and 9 days before experiment 1, 2 and 3, respectively. This period was needed to ensure a depletion of the oxygen concentration which entered the soil during the transfer. The plants used in the first experiment were 106 days, in the second 93 days and the third 112 days old, respectively. Experiments were performed at 25 ± 1.5 °C with 12 hours photoperiods. The water level on top of the soil was maintained constant by daily addition.

Experimental set-up

The plants used for experiments were selected from the breeding trays. The glass jar (diameter of 21 cm, height of 30 cm) is divided in two parts by a glass-sintered separation (thickness of 0.8 cm, diameter of 15 cm, pore size of 100–160 μm, porosity of 0.4 by volume). The top part (height of 25 cm) is open. A single rice plant was carefully placed inside the top of the jar, without damaging the structure of the soil and roots during the transfer from the plastic trays (Figure 2). The bottom part of the jar represents the injection reservoir (volume $V_r = 2.39\ cm^3$) which contains water and where $^{13}CH_4$ can be injected. The separation allows $^{13}CH_4$ diffusion from the reservoir into the top part of the jar, and prevents movement of gas bubbles to and from the reservoir. A Bell glass jar placed over the plants (diameter of 10 cm, height of 75 cm) formed a headspace that was sealed at the bottom by a water layer on top of the soil. Before placing the plants the reservoir was carefully filled with cold boiled water (to reduce dissolved gases). During experiments a magnetic stirrer continuously stirred the water in the reservoir.

Six ml of $^{13}CH_4$ gas was injected into the reservoir of the jar containing one rice plant, the so-called 'enriched' plant. The injected amount was far below the maximum solubility of methane in water (Henry coefficient at 20 °C for CH_4 is $k_H = 1.3 \times 10^{-3}$ mol $kg(H_2O)^{-1}$ bar^{-1}, (Borchers et al. 1969). Before injection, samples of CO_2 and CH_4 were taken to determine the background $^{13}C/^{12}C$-isotope ratio. As control we used a second 'reference' plant set-up, similar to the first one, except the fact that CH_4 was not added.

Trace gases emitted by the plant were transported by a carrier gas to the monitoring and sampling circuit lines (Figure 3). As carrier we used air at continuous flow of 3 l/h (φ_{car}). The gas inlet was placed at the bottom of Bell jar and the outlet at the top. To reduce condensation on the glass walls, the air was circulated in a closed circuit through a water condenser by a small pump and a heating wire was wrapped around the Bell jar (Figure 2). To provide enough CO_2 for photosynthesis during the light periods, the air was enriched with CO_2 up to a concentration of about 0.1%. After passing the plant, the airflow was split into two parts. One part

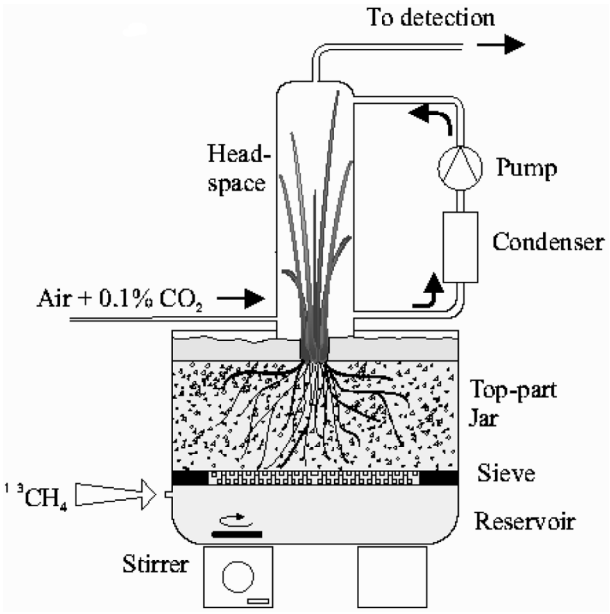

Figure 2. Experimental soil-plant system. The rice plant with soil was placed into the top part of the glass jar. The lower part of the jar consists of a reservoir. The reservoir is separated from the upper part by a sintered glass ″sieve″. The reservoir was filled with degassed water to which $^{13}CH_4$ methane is added and which is stirred by a magnetic stirrer. A Bell glass jar was placed over the plant forming a headspace for collecting the emitted gases. It was sealed at the bottom by a water layer on top of the soil.

(0.5 l/h) was used for on-line monitoring of the total CO_2 and CH_4 concentrations (monitoring lines in Figure 3). The rest (2.5 l/h) was continuously flushed through the sampling bottles for the off-line isotope analysis (sample lines in Figure 3). The gas flow was controlled by electrical mass flow controllers (type Brooks 5850). The CO_2 and CH_4 concentrations were determined from the isotope enriched plant and the reference plant, which were alternately connected to the monitoring lines by computer-controlled valves. CO_2 and CH_4 concentrations were measured on-line using a non-dispersive infrared detector (type Hartmann & Braun, Uras 10E) with a detection range from 0 to 800 ppm for CH_4, and from 0 to 300 ppm for CO_2 (accuracy 1% of the span). Because the CO_2 concentrations in the headspace around the plants were about 10 times higher than the upper detection limit of the instrument, the gas flow was diluted with nitrogen at a controlled ratio. For the off-line isotope ratio analyses each sample line contained two glass bottles (volume of 2.5 l each); one for sampling CO_2, one for CH_4. Water vapour was removed with a $CaCl_2$ scrubber from the airflow before entering the CO_2 bottle. After passing through the CO_2 bottle, the air was scrubbed of CO_2 by aqueous NaOH. Next, methane was oxidized to CO_2 by a Cu(II)O catalyser (″Cat.″ in Figure 3) and the airflow was flushed through the CH_4 sampling bottle. The oxidation step has been tested for complete oxidation. However, even incomplete oxidation would have led

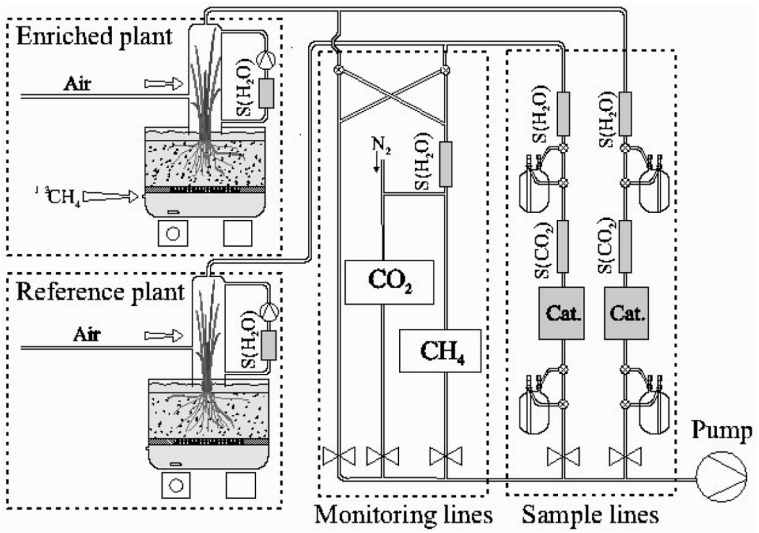

Figure 3. Experimental set-up for the methane oxidation experiments. Part of the gases emitted by the enriched and reference rice plants (0.5 l/h) is transported to the monitoring lines for on-line measurements of the total CO_2 and CH_4 concentrations. The rest of the gas emission (2.5 l/h) is continuously flushed through the sampling bottles for the off-line isotope analysis. Scrubbers are indicated as S(H_2O) for water and as S(CO_2) for CO_2. The boxes indicated with 'Cat.' are the catalysers used to oxidize CH_4 into CO_2.

to a relatively small and negligible shift in the CH_4 isotope signal because we used labelled $^{13}CH_4$, and other errors would have prevailed (see the section of isotope ratio measurement). The amount of CO_2 resulting from methane oxidation will be indicated as ″CH_4-sample″. Note that methane was not removed from the air in the CO_2-samples. Methane has a low boiling point compared to CO_2 and therefore can easily be separated from CO_2 during the preparation steps for the isotope ratio determination. A Viton o-ring high vacuum tap (type Louwers-Hapert) was used as inlet and outlet for each bottle. The bottles were automatically closed each morning, half an hour before the light period started, using electric valves. A 2.5 kW electric oven (type Philips, set at 1000 °C) was used to heat the quartz tube containing the catalyst. The inner diameter of the oven was 7 cm (height of 49 cm) and filled with sand to provide a good temperature transfer to the catalyser. The volume of the quartz tube was 50 ml and filled with sintered Cu(II)O (Merck, pro analysis). Sintered Cu(II)O was obtained by heating the Cu(II)O to 1055 °C.

Isotope ratio measurement

The isotope ratios were determined (off-line) at the Centre for Isotope Research (CIO), University of Groningen (the Netherlands). The CO_2 was extracted from air in a sample bottle using a circulation set-up. In this set-up, water was extracted from the air using dry ice (195 K), while CO_2 was cryogenically collected using a

liquid N_2 trap (80 K). The system has been designed for atmospheric CO_2 studies which requires very high accuracy (of 0.02‰) (Wildschut 2001; Meijer et al. 2000). The CO_2 samples were analysed with a Micromass SIRA 9 (for the CO_2 samples) and a Micromass OPTIMA (for the CH_4 samples) dual inlet isotope ratio mass spectrometer. The internal machine precision of the SIRA 9 was 0.02‰. It required at least 1 ml of pure CO_2 gas under standard conditions of temperature and pressure (STP). The OPTIMA could handle samples down to 0.1 ml CO_2 gas (at STP) with an internal precision smaller than 0.01‰. Due to the high isotope ratios for methane, the error in the calculated values is caused mainly by inaccuracies and non-linearity of the calibration curve of the instrument (Meijer et al. 2000). The absolute error of our measurements is estimated at about 0.05‰ with a relative error of 0.2% for the measured value.

Carbon isotope ratios are expressed as $\delta^{13}C$ (‰) values, relative to the standard isotope ratio, Vienna Pee Dee Belemnite (VPDB) (Gonfiantini 1983):

$$\delta^{13}C = \frac{(^{13}C/^{12}C)_{sample}}{(^{13}C\,^{12}C)_{VPDB}} - 1 \tag{1}$$

where $(^{13}C/^{12}C)_{sample}$ is the carbon isotope ratio in the sample and $(^{13}C/^{12}C)_{VPDB}$ the standard ratio (defined to be 0.0112372). Using Equation (1), the concentration difference of ^{13}C emission ($[^{13}C]_{net}$) between the enriched plant ($[^{13}C]_{enr}$) and the reference plant ($[^{13}C]_{ref}$) is given by:

$$[\,^{13}C]_{net} = [C]_{enr}(\,^{13}C/\,^{12}C)_{VPDB}(\delta\,^{13}C_{\,enr} - \delta\,^{13}C_{\,ref}) \tag{2}$$

assuming $[^{12}C]_{enr} \approx [^{12}C]_{ref} \approx [C]_{enr} \approx [C]_{ref}$. As can be seen from Equation (2), the net concentration difference of $^{13}CO_2$ (or $^{13}CH_4$) is determined as the product of the total CO_2 (or CH_4) concentration (determined on-line) and the difference of the measured isotope ratios $\delta^{13}C_{enr}$ and $\delta^{13}C_{ref}$ (from off-line samples), multiplied by the VPDB standard isotope ratio. The difference of isotope ratios between the enriched and the reference plant ($\delta^{13}C_{enr}$–$\delta^{13}C_{ref}$) was calculated for each pair of data points (see e.g. Figure 5). In this way, trends in the measured reference isotope ratios over time were corrected. The initial difference in $\delta^{13}C$-values between the plants (first point) is added or subtracted in order to have a zero emission before the point corresponding to the $^{13}CH_4$ injection. Finally, we determined the oxidation fraction from:

$$\text{Oxidation}\quad\text{fraction} = \frac{[\,^{13}CO_{\,2}]_{net}}{[\,^{13}CO_{\,2}]_{net} + [\,^{13}CH_{\,4}]_{net}} \tag{3}$$

$^{13}CO_2$ exchange with CO_2 in the soil

$^{13}CH_4$ can be partly oxidized to $^{13}CO_2$ in the rhizosphere around the roots of the plants. In contrast to methane, CO_2 is strongly soluble in water and $^{13}CO_2$ is exchanged with a large buffer of CO_2 dissolved in the soil pore water. Therefore, only part of the produced $^{13}CO_2$ will be emitted to the atmosphere. This results in a lower ($^{13}CO_2/^{12}CO_2$) emission ratio into the atmosphere than that of CO_2 present in the rhizosphere. The difference between $^{13}CH_4$ and $^{13}CO_2$ emission can cause the underestimation of the oxidized fraction for $^{13}CH_4$ in the rhizosphere, especially for short periods. When the CO_2 buffer in the soil water reaches steady state, $^{13}CO_2/^{12}CO_2$ represents the isotope ratio in the rhizosphere. To investigate the influence of the CO_2 buffer on the oxidation ratios calculated from Equation 3, we used a simple model to describe the buffering process. The diffusion of $^{13}CO_2$ from $^{13}CH_4$ oxidation is limited to the rhizosphere. Therefore the size of the buffer volume is equal to that of the rhizosphere around the roots. In this exchange volume, V_{ex} (m^3), the CO_2 isotope ratio will increase exponentially to a steady state value. The diffusive exchange of CO_2 with other parts of the soil is neglected. In addition, we assumed a constant $^{13}CO_2$ production due to oxidation, noted by p_{CO2} (m^3/s). At these conditions, the time-dependent $^{13}CO_2$ emission, indicated by $F_t(^{13}CO_2)$ is expressed by:

$$F_t(^{13}CO_2) = F_{\max}(1 - e^{-t/\tau}) \tag{4}$$

where F_{max} represents the steady state value (m^3/h) and $\tau = V_{ex}/p_{CO2}$ (s) is the time necessary to reach the steady state. The values for τ and F_{max} were used as parameters to fit this model with the measured values.

During the experiment, CO_2 and CH_4 production emitted by the plants were transported by the carrier flow, φ_{car} (m^3/h), through the headspace. The flow from the plant is negligible compared to the carrier gas flow, which therefore determines the gas flow at the exit of the headspace:

$$\phi_{car}[CO_2]_{car} + F_t(^{13}CO_2) = \phi_{car}[CO_2]_m \tag{5}$$

$[CO_2]_m$ (m^3/m^3) is the measured concentration and $[CO_2]_{car}$ is the CO_2-concentration in the carrier flow before it entered the headspace. The air entering the headspace contains CO_2 with naturally abundant $^{13}CO_2$ for both the enriched plant and the reference plant. We have measured this value to be around −29.87‰. Since in Equation (2) we consider the difference between $\delta^{13}CO_2$ of the enriched and the reference plant, the $^{13}CO_2$ flow out of plant is free of the carrier gas contribution and it can be expressed by:

$$F_t(^{13}CO_2) = \phi_{car}[^{13}CO_2]_{net} \tag{6}$$

$[^{13}CO_2]_{net}$ can be determined from Equation (2). This model is simplified, since we assume a constant $^{13}CO_2$ production in the rhizosphere. Practically, the produc-

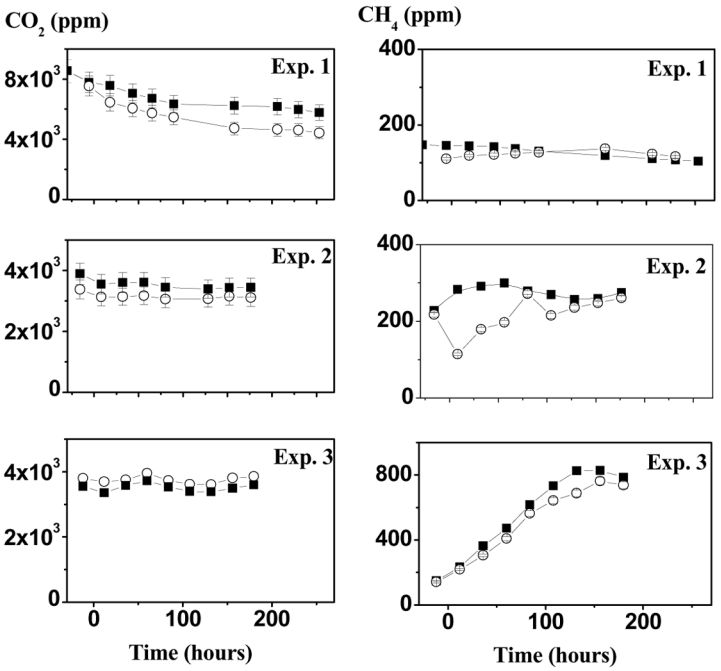

Figure 4. Total CO_2 and CH_4 emission of the enriched plant (■) and reference plant (○) during experiment 1, 2 and 3. The CO_2 concentrations were determined at the end of the night period. During day time the CO_2 concentration dropped to zero due to photosynthesis. $^{13}CH_4$ is injected at t = 0.

tion of $^{13}CO_2$ depends on the $^{13}CH_4$ concentration. Initially, before the injection, the $^{13}CH_4$ concentration is zero and increases over time, according to the $^{13}CH_4$ diffusion through the soil.

Results

Oxidation fraction

Figure 4 shows the total on-line emission of CO_2 and CH_4 from the enriched and reference plants respectively, for each of the three experiments. Pre-dawn CO_2 concentrations remained constant throughout the experiment, indicating that the plants remained healthy. In the third experiment, a significant rise in the CH_4 concentration was observed. CO_2 and CH_4 (Figure 5) showed a clear $\delta^{13}C$ enrichment in the first two experiments (1 and 2). The third experiment showed a much smaller difference in the CO_2 isotope ratio; however, the methane enrichment was as high as in the other experiments.

The [$^{13}CO_2$] and [$^{13}CH_4$] concentrations calculated from Equation (2) are shown in Figure 6. The oxidation fractionations calculated using Equation (3) are presented

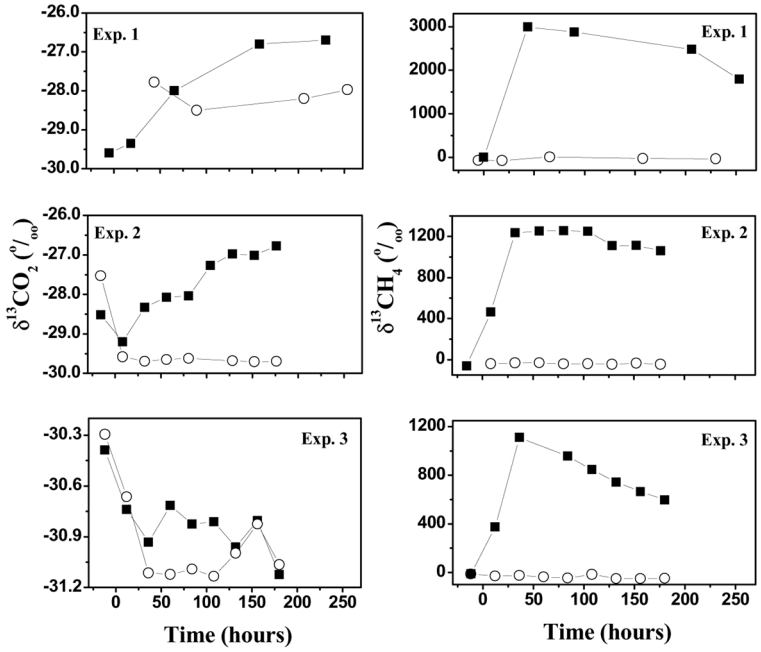

Figure 5. $\delta^{13}CO_2$ and $\delta^{13}CH_4$ values corresponding to the enriched plant (■) and the reference plant (○) as compared to the VPDB-standard ratio (‰). Data are shown for three experiments.

in Table 2. The oxidation fraction was about 5% in experiment 1 and 2, but experiment 3 basically gave 0% oxidation. Failure to observe an oxidation fraction must be attributed entirely to the very low difference in $\delta^{13}CO_2$ values between the enriched and reference plant. All other gas concentrations had similar values to those in the first two experiments, which proves that the low oxidation fraction is not an artefact in the experimental set-up. The accuracy of the oxidation fraction depends mainly on the accuracy of the on-line data monitoring of CO_2 and CH_4 concentrations, being 3% for both. The precision in the isotope ratio calculation is very high and, therefore, did not contribute to the total error. However, the largest inaccuracy for $^{13}CO_2$ determination was caused by the variation of the $^{13}CO_2$ level. This reference level was subtracted point-wise using Equation 2 (see Figure 5). An alternative subtraction, such as averaging all $\delta^{13}C$-values of the reference plant, gave only a slightly different result. The $^{13}CH_4$ concentration was fully determined by the error in the total concentration calculation and, due to the high $\delta^{13}CH_4$ values, it was not affected by the fluctuation in the reference plant. Finally, the error in the oxidation fraction determination becomes 4%.

Origin of the $^{13}CO_2$

From Figure 6 it can be seen that in experiments 1 and 2, $^{13}CH_4$ reached steady state after about 50 h, while the $^{13}CO_2$ concentration continued to increase even

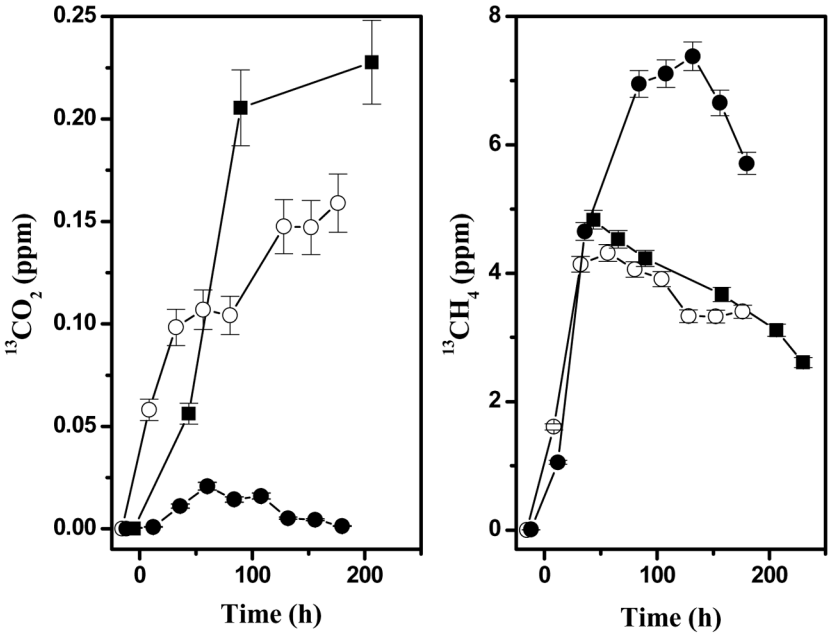

Figure 6. The trend of $^{13}CO_2$ and $^{13}CH_4$ concentrations during experiment 1 (■), 2 (○) and 3 (●). $^{13}CH_4$ was injected at t = 0.

Table 2. Oxidation fractions of $^{13}CH_4$ of the three experiments (column 1). The integrated amount of the emitted $^{13}CH_4$ during the experiment as a fraction of the injected amount (column 2). Values for F_{max} and τ (column 3 and 4) determined for experiments 1 and 2 using the CO_2 exchange model. F_{max} represents the $^{13}CO_2$ emission rate corresponding to steady state. The trend of the third experiment could not be fitted.

	Oxidation fraction from $^{13}CH_4$ (%)	$^{13}CH_4$ (%)	F_{max} ($\times 10^{-10}$ m_3/h)	τ (hours)
Experiment 1	6.8 ± 0.3	31.8	8 ± 3	94 ± 70
Experiment 2	4.5 ± 0.2	30.6	4.4 ± 0.4	34 ± 11
Experiment 3	0.02 ± 0.001	51.0		

after 200 h and did not reach the steady state. After 50 h, $^{13}CH_4$ emission declined due to depletion of added $^{13}CH_4$. Microbial growth, triggered by the applied $^{13}CH_4$, cannot explain the slower rise in $^{13}CO_2$, because the relative contribution of the $^{13}CH_4$ tracer to the total CH_4 concentration in the soil is small (compare Figure 4 and Figure 6B). This delay in formation of $^{13}CO_2$ must be attributed to exchange of CO_2 generated by the bacteria with CO_2 dissolved in the soil. Equation 4 describes this behaviour and, by fitting this model to the experimental data, we determined F_{max} and τ. The values of F_{max} and τ are presented in column 3 and 4 of Table 2 and the fits to the experimental curves are shown in Figure 7.

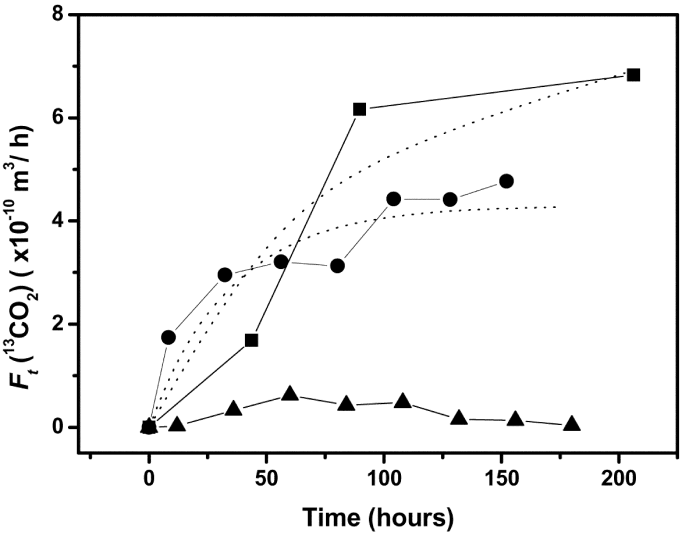

Figure 7. $^{13}CO_2$ emission measured in experiment 1 (■), 2 (●) and 3 (▲) and fitted with the CO_2 exchange model (solid lines). The third experiment could not be fitted. The values used for F_{max} and τ are presented in Table 2.

We showed for experiment 3 no fit, because emissions were order of magnitude lower and because of the delay in the emission, which cannot be described by the model. The $^{13}CO_2$ emission measured at the end of the experiments 1 and 2 appears to be almost in steady state; the values are at 88% and 99% of the calculated values of F_{max}, respectively.

Discussion

The $^{13}CH_4$ emitted in the headspace of the plants during the experiment was found to be 30–50% of the amount injected at the start of the experiment (Table 2). The depletion of the reservoir is significant and cannot be explained by diffusion through thicker layers of soil. The diffusion of methane through water saturated soil depends on its diffusion coefficient through water (1.83×10^{-5} cm^2/s) (Borchers et al. 1969). For a 15 cm thick soil only extremely small portions of the isotopically labelled methane will reach the top level of the soil. In addition, for diffusion processes the ″sieve″ is equivalent to a 0.8 cm thick soil layer. Applying Fick's diffusion law to the $^{13}CH_4$ flux in Figure 4, we estimated the average soil thickness before reaching the rhizosphere around the roots to be 12 ± 8 mm, 22 ± 4 mm and 39 ± 5 mm for experiment 1, 2 and 3 respectively. If the ″sieve″ thickness of 0.8 cm is taken into account, it can be seen that the methane could have reached only the roots close to the ″sieve″. The measured oxidation fractions need to not be corrected for the fraction of the roots accounted for, because $^{13}CH_4$ did not reach the higher roots either. Methane produced around the higher roots by methanogenic

bacteria and oxidized to CO_2 are subtracted as background in both treatments, which is an important advantage of the isotope labelling approach. CO_2 added at daytime to the headspaces for plant photosynthesis could have influenced the $\delta^{13}C$ values which were monitored daily. However, because both reference plant and enriched plant were supplied with extra CO_2 and the difference in their $\delta^{13}C$ were considered in the calculation of the methane oxidation fraction, this shift in background level does not affect our estimated methane oxidation fractions. Moreover, isotope ratio samples were taken at night to avoid fractionation by photosynthesis. The measured oxidation fractions for experiment 1 and 2 were 4.5% and 6.8%, respectively, while for the third experiment it was below the detection limit. In experiment 3 the difference in $\delta^{13}CO_2$ emission between the enriched and the reference plant was much smaller than in experiment 1 and 2 (Figure 5), resulting in an almost zero oxidation fraction. Because methane during experiment 3 was high and emission of the injected isotope labelled methane was comparable to that in experiments 1 and 2 (Figure 6B), the low oxidation fraction in experiment 3 must be attributed to a 'real' low oxidation rates by methanotrophic bacteria.

The percentage of the methane oxidation presented in this study is lower than that in most earlier studies. However, our experiments refer to about 100 days after flooding the soil, at a stage when similarly low oxidation fractions were also reported by Denier van der Gon and Neue (1996) and Krüger et al. (2001) who used an inhibition technique (see Table 1). The inhibition technique is the least problematic of the approaches used in those earlier methane oxidation studies. van Bodegom et al. (2001) showed that over time, root growth reduced the predicted methane oxidation fraction from 33% to 8.5%, arriving at oxidation estimates similar to those in this study. Based on these comparisons, there is no reason to doubt the validity of the method applied here, even though our estimates are much lower than those of previous work.

The low oxidation fraction suggest that oxidation is not an important reason for the variability in the measured methane emission from rice paddy fields during the latter part of the growing season. The methane production itself may cause this variation. The question why methane oxidation decreases during the later part of the growing season remains still to be clarified. Bodelier et al. (2000) showed that methane oxidation in flooded rice paddies is often limited by nitrogen availability, most likely caused by intensive competition for N between rice plants and heterotrophic bacteria. Dan et al. (2001) showed that after fertilisation nitrogen is available only for a few days. In addition to a nitrogen limitation, oxygen may become limiting for methanotrophs at the end of the growing season: Satpathy et al. (1997) showed that the root oxidizing power of rice plants decreased during the latter part of the season. Using inhibition techniques, highest methane oxidation estimates are observed in the first part of the growing season. E.g. for young plants (30 days after flooding) Krüger et al. (2001) found an oxidation fraction of 40%. Apparently, methane oxidation may be relatively important process in young plants

Conclusions

Methane oxidation fractions in the rhizosphere reported so far in literature show large variations (Table 1). Those obtained using CH_3F, CH_2F_2 or C_2H_2 as inhibitors appear to be most trustworthy, yielding the most consistent values, in the range of 0–52%, depending on the date in the growing season. Oxidation fractions tend to decrease in the course of the growing period. However, these results can be influenced by unwanted side effects of the inhibitor on methanogenic bacteria. Here we present a new method, that does not suffer from the various artefacts associated with the other methods. It is based on the addition of ^{13}C-labelled methane and subsequent detection of the $^{13}C/^{12}C$ isotope ratio of both methane and CO_2 in the headspace around the rice plants. The methane oxidation fractions in rhizosphere during the latter part of the growing season were found to be in the range of 0–7%. We conclude that the fraction of methane that is oxidized in the complete soil-plant system are quite low during the latter part of the growing season, which is probably due to a combination of nitrogen and oxygen limitation to the methanotrophs.

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